Identification of Human Lymphoma Cells by Antisera to Malignancy-Associated Nucleolar Antigens

By Richard J. Ford, Michael Cramer, and Frances M. Davis

The non-Hodgkin's lymphomas (NHL) are a diverse group of human lymphoid neoplasms that have long presented pathologists with formidable diagnostic challenges. These tumors of the immune system are thought to represent neoplastic transformations of most of the recognized stages in T and B lymphocyte ontogeny. Lymphoma cells, however, often simulate their normal lymphocytic counterparts both morphologically and cell surface phenotypically, creating difficulties in discriminating normal from neoplastic lymphocytes. We have used heteroantisera to the human malignancy-associated nucleolar antigen (HMNA) to prospectively evaluate its efficacy in identifying the morphologically neoplastic cells in NHL lesions. In 65 cases of T and B cell histopathologic types of NHL, the antisera reacted with nucleoli in the morphologically and cytogenetically neoplastic lymphoma cells, but not with normal-appearing lymphoid and other cell types present in the lesions. Control specimens from normal and hyperplastic lymphoid tissue also failed to react with anti-HMNA antibodies. Normal activated lymphoid cells in vitro and growth-factor-dependent normal lymphoid cell lines also failed to express the nucleolar antigen(s). These data suggest that the HMNA is a valuable tumor cell marker for neoplastic human lymphoid cell populations and can be used with other types of cell markers for a better definition of the neoplastic cells in NHL.

**THE NON-HODGKIN'S LYMPHOMAS** (NHL) have long presented pathologists with a diagnostic challenge. These neoplasms of the human immune system are thought to represent malignant transformations of lymphoid cells involved in immune reactions. The spectrum of human lymphoid neoplasia is wide, representing, in the NHL, most if not all of the putative stages in normal lymphoid differentiation with neoplastic counterparts. The histopathologic diagnosis of NHL has traditionally relied on effacement of lymphoid organ structure, pattern of tissue involvement, and cellular atypia. These criteria, due to their subjective nature, have often led to confusion in the identification and classification of these neoplasms. Other difficult interpretive problems arise when nonneoplastic reactive lesions simulate malignant lymphomatous processes. These problems have been aided in the B cell NHL by the recent development of immunoglobulin light-chain typing, which can be used for identifying the monoclonal neoplastic lymphoid cell populations. The identification of monoclonal cell populations suggests that the majority of the cells represent the neoplastic clone, but it does not differentiate the neoplastic cells from residual normal B cells of the same light-chain type, which requires antidiagnostic agents. Normal B cells comprise a variable percentage of the nonneoplastic cells in these lesions. Another major criterion for malignancy in lymphoid neoplasms is cytogenetic abnormality, which is demonstrable by karyotypic and Giemsa banding techniques.

The identification of a human malignancy-associated nucleolar antigen(s) (HMNA) has provided a means of identifying neoplastic cells expressing this antigen(s) by standard immunologic methods. These authors have studied a variety of human tumor types, including hematologic tumors, and have found that the HMNA identifies tumor cell populations selectively. No study to date, however, has evaluated the efficacy of the HMNA as a diagnostic tumor cell marker in human lymphoid neoplasms.

In this study, we have used heteroantisera to HMNA in conjunction with morphological and cell surface marker analysis, including studies with monoclonal antibodies, to evaluate the ability of such antisera to differentiate neoplastic lymphoid cells from reactive or normal lymphocytes. The results of this prospective study indicate that the HMNA is a useful tumor marker in the NHL. HMNA identification, combined with cell surface phenotypic and morphologic histopathologic analysis, can provide a more complete assessment of the neoplastic cell populations present in a lymphomatous lesion.

**MATERIALS AND METHODS**

**Patient Population**

Surgical biopsy specimens from patients with known or suspected lymphoma were obtained fresh at the time of biopsy procedure. These specimens consisted usually of lymph nodes, spleens, or soft tissue masses, but also included biopsies from the gastrointestinal tract and skin. Both adult and pediatric cases were included. Control tissue included histologically uninvolved lymph nodes from mastectomy and colectomy specimens, hyperplastic (reactive) lymph nodes, as well as nonstimulated and mitogen [phytohemagglutinin (PHA), pokeweed mitogen (PWM)] activated peripheral blood lymphocyte lineages. The non-Hodgkin's lymphomas are a diverse group of human lymphoid neoplasms that have long presented pathologists with formidable diagnostic challenges. The identification of human malignancy-associated nucleolar antigen(s) (HMNA) has provided a means of identifying neoplastic cells expressing this antigen(s) by standard immunologic methods. These authors have studied a variety of human tumor types, including hematologic tumors, and have found that the HMNA identifies tumor cell populations selectively. No study to date, however, has evaluated the efficacy of the HMNA as a diagnostic tumor cell marker in human lymphoid neoplasms. In this study, we have used heteroantisera to HMNA in conjunction with morphological and cell surface marker analysis, including studies with monoclonal antibodies, to evaluate the ability of such antisera to differentiate neoplastic lymphoid cells from reactive or normal lymphocytes. The results of this prospective study indicate that the HMNA is a useful tumor marker in the NHL. HMNA identification, combined with cell surface phenotypic and morphologic histopathologic analysis, can provide a more complete assessment of the neoplastic cell populations present in a lymphomatous lesion.

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populations. Normal thymus and hyperplastic tonsils were also examined, as were activated human peripheral blood T cells grown for greater than 21 days in T cell growth-factor-dependent culture.

**Tissue Preparation**

Freshly obtained neoplastic or normal lymphoid tissue was examined grossly, and appropriate sections were taken for frozen section. Frozen thin (3-5 μm) sections were cut on a Harris glove box cryostat. These sections were immediately fixed and stained with hematoxylin and eosin (H&E), and adjacent sections were fixed in cold methanol at 20°C for 10 min for immunofluorescence.

**Preparation of Antiserum to Human Malignancy-Associated Nucleolar Antigen**

The antisera were prepared according to the method described by Davis et al. Briefly, New Zealand white (NZW) rabbits were injected with isolated whole HeLa cell nucleioli intradermally and subcutaneously. The nucleioli were prepared using standard cell fractionation procedures including hypotonic shock, NP-40 detergent treatment, Dounce homogenization, sonication, and differential sedimentation through sucrose. The IgG fraction was prepared from the antisera by 50% (NH₄)₂SO₄ fractionation and chromatography on DEAE-Sephacel. The IgG fraction was then absorbed with fetal calf serum and sonicated placental nuclei, as previously described. 

**Immunofluorescence**

Indirect immunofluorescence was performed on fixed cryostat sections using the IgG fraction of the rabbit anti-HMNA serum, and FITC-labeled goat anti-rabbit IgG (Miles, Elkhart, IN) was used as the second antibody. The sections were examined on a Leitz Orthoplan fluorescence microscope equipped with a Ploem illuminator. Correlations were then made with adjacent frozen sections stained with hematoxylin and eosin and with the permanent paraffin-embedded H&E sections for each case.

**Cell Surface Markers**

Conventional cell surface immunologic markers were performed on single cell suspensions of lymph node, spleen, and other biopsy specimens. These markers included sheep red cell rosettes (En), cell surface immunoglobulin (Slg) for various isotypes using F(ab')₂-fluorescein (FITC) conjugated antisera, and in some cases, assays for lymphocytes bearing complement receptor (CR). The assays were performed as previously described.

**Monoclonal Antibody Phenotyping**

Single-cell suspensions from lymph nodes and other tissues were stained with a variety of commercially available monoclonal antibodies, either directly with FITC-conjugated antibodies or by an indirect double-antibody method. The antibodies used were titrated by staining purified populations of normal peripheral blood T and B lymphoid cells. The stained cell preparations were then evaluated for antigen expression on a Spectrum III cytometer (Ortho Diagnostics, Raritan, NJ). The monoclonal antibody profile from cell suspensions was then compared with monoclonal antibody-stained frozen sections of the tumor-involved or control tissues to correlate antigen expression with the pattern of tumor cell involvement in the lymphoid tissue specimen.

**In Vitro Cell Culture Studies on Normal Lymphoid Cells**

Normal peripheral blood T and B cells were activated in vitro by lectin stimulation with 1.0% (w/v) phytohemagglutinin (PHA, Difco, Detroit, MI), or 1.0% w/v pokeweed mitogen (PWM, GIBCO, Grand Island, NY), respectively, for 72 hr in RPMI and 10% fetal calf serum. The T and B blasts were washed, and cytocentrifuge (Shandon, Sewickley, PA) preparations were made and fixed in absolute methanol at 20°C for 10 min. Mixed lymphocyte cultures (MLC) from two donor peripheral blood mononuclear cell (PBMC) preparations were harvested after 96 hr and prepared in an identical manner. In addition, polyclonally activated T cell lines from PBMC were maintained on T cell growth factor (TCGF) containing supernatants for at least 3 wk prior to assay. Cytocentrifuge preparations from these factor-dependent cell lines were fixed and assayed for HMNA as described. 

**Cytogenetics**

Frozen lymphoma cells from biopsy specimens were cell surface phenotyped, and the tumor cell population (Slg+ or En+ cells in most cases studied) was enriched for either negative or positive selection using En rosetting. Macrophages and other adherent accessory cells were removed by adherence to Petri dishes for 1 hr at 37°C. The tumor-cell-enriched population was then prepared for cytogenetic analysis. This same population was also assayed for HMNA on fixed cytoseifuge preparations for correlation of karyotype with the presence of HMNA, as well as on Giemsa-stained cytocentrifuge preparations for morphological correlation. 

**Histopathologic Correlation With HMNA**

Frozen tissue sections from lymph nodes or other lymphomatous specimens that represented adjacent sections to those assayed for HMNA were stained with H&E and compared to both the fluorescent-stained HMNA section and with the paraffin-embedded section from the same specimen. Two hematopathologists independently reviewed the sections for correlation of HMNA-positive cells with the tumor population, using the standard morphologic criteria previously mentioned. The paraffin sections of the same specimen were also reviewed and compared with respect to tumor cell type and diagnostic classification with results obtained by a third independent hematopathologist who was not involved in this study.

<table>
<thead>
<tr>
<th>Lymphoma Type*</th>
<th>Number of Cases</th>
<th>Number of HMNA+ Cases†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well differentiated lymphocytic (WDL)</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Nodular poorly differentiated lymphocytic (N-PDL)</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Undifferentiated, Burkitt's type</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Lymphoblastic (&quot;convoluted&quot;)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Diffuse large cell—B cell type</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Diffuse large cell—T cell type</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Diffuse poorly differentiated—T cell type</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Cutaneous T cell (mycosis fungoides)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Nodular large cell—B cell type</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Mixed, small and large cell—B cell type</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Hairy cell—B cell type</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>65</td>
<td>65</td>
</tr>
</tbody>
</table>

*Modified Rappaport classification, determined on paraffin-embedded, H & E-stained 6-μm tissue sections.
†Reactivity determined by rabbit anti-HMNA antiserum with correlation of the HMNA+ areas and the tumor-involved areas in the tissue by histopathologic methods; HMNA varied from 60%–>90% of the putative tumor cells, depending on the type of lymphoma and the pattern of infiltration in the lymphoid tissue.
RESULTS

Identification of HMNA in Human Lymphoma Cells

Sixty-five cases of a representative spectrum of NHL from a wide range of anatomical sites were studied with heteroantisera to HMNA. The majority of these cases were diagnostic lymph node biopsies where the nodes or tissues were replaced by, or infiltrated with, morphologically neoplastic cells, as observed on the initial frozen section sampling of the specimen. The results of indirect immunofluorescent staining for HMNA on adjacent frozen sections in these cases is shown in Table I, where it can be seen that the antisera identified nucleoli in cells corresponding to the morphologically malignant cells in each of the 65 cases studied. The staining pattern observed was a punctate intranuclear fluorescence, which is consistent with nucleolar localization (Fig. 1, C and D). The pattern of positive nucleolar fluorescent staining was observed to correspond to the pattern of neoplastic cell infiltration. Simultaneous phase-contrast and fluorescence microscopic examination of these sections confirmed the nucleolar localization of the fluorescence observed. The area of fluorescence within the nucleus of the neoplastic cells varied both in size and in fluorescence intensity, generally being most prominent in the large cell lymphomas, but also clearly demonstrable in the small cell histopathologic types (Fig. 1C). Cytocentrifuge preparations from lymphoma cell suspensions also clearly revealed the presence of nucleolar-associated fluorescence when assayed with the anti-HMNA antisera (Fig. 2, A, C, and E). The

![Image](image-url)
cells is, of course, of central importance. In addition to the absence of HMNA in histologically normal-appearing cells, often seen infiltrating or surrounding morphologically neoplastic lymphoma cells, we also examined a series of normal lymph nodes removed from surgical specimens, including mastectomy, colectomy, and radical neck dissection specimens. As with the diagnostic lymphoma lymph node biopsies, these nodes were histologically examined immediately by frozen section and later in paraffin sections to assure that no morphologically demonstrable tumor cells were present. As seen in Table 2, none of the normal lymph nodes or other normal lymphoid tissue examined was found to reveal the presence of HMNA in the lymphoid or other cells present. Diagnostic biopsies from cases of lymphadenopathy, showing only follicular or generalized lymphoid hyperplasia ("reactive" nodes), were uniformly negative, except in one case of a patient with an AIDS-like syndrome with diffuse persistent lymphadenopathy, where 30%–40% of lymphoid cells expressed HMNA. The histopathology in this case revealed hyperplastic, but not neoplastic, morphological features, and a monoclonal cell population could not be identified by light-chain typing.

**Cell Surface Phenotyping and Cytogenetics**

To better characterize the putative lineage of the NHL cells and to further confirm the association of HMNA with neoplastic lymphoid cells, the putative tumor cells were separated from accessory cells present in the lesions by En rosetting and removal of adherent cells. The resulting positively (T cell type) or negatively (B cell type) selected tumor cell populations were characterized by monoclonal antibodies to determine the cell surface phenotype. The separated cell

**Absence of HMNA in Normal Human Lymphoid Cells**

The importance of demonstrating the absence of a putative tumor cell marker in normal or nonneoplastic
were found to enrich for HMNA-positive cells to
enrich preparations. These separation procedures
were found to express the
populations were then assayed for HMNA on cytocen-
the HMNA-expressing cell populations, cytogenetic
NHL expressing a variety of cell surface phenotypes,
positive cells. Table 3 shows representative NHL cases
of the total viable cells present. The other
indicates >60% of the cells showing nucleolar fluorescence within the morphological tumor cell population.

Table 3. HMNA Correlation With Conventional Markers and Monoclonal Antibody Phenotypes

<table>
<thead>
<tr>
<th>Lymphoma Type*</th>
<th>Conventional Markers</th>
<th>Monoclonal Antibody†</th>
<th>HMNA‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>En</td>
<td>Sig</td>
<td>clg</td>
</tr>
<tr>
<td>(A) Small cell type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-PDL</td>
<td></td>
<td>μδχ</td>
<td></td>
</tr>
<tr>
<td>N-PDL</td>
<td></td>
<td>μγχ</td>
<td></td>
</tr>
<tr>
<td>N-PDL</td>
<td></td>
<td>μδχ</td>
<td></td>
</tr>
<tr>
<td>D-PDL</td>
<td></td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>D-PDL (Skin)</td>
<td></td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>D-PDL</td>
<td></td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Burkitt’s</td>
<td></td>
<td>μχ</td>
<td></td>
</tr>
<tr>
<td>HCL</td>
<td></td>
<td>μδχ</td>
<td></td>
</tr>
<tr>
<td>D-WDL</td>
<td></td>
<td>μδλ</td>
<td></td>
</tr>
<tr>
<td>D-WDL</td>
<td></td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(B) Large cell type</td>
<td></td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>D-LCL</td>
<td></td>
<td>μχ</td>
<td></td>
</tr>
<tr>
<td>D-LCL</td>
<td></td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>D-LCL</td>
<td></td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>D-LCL</td>
<td></td>
<td>μχ</td>
<td></td>
</tr>
<tr>
<td>N-LCL</td>
<td></td>
<td>μδχ</td>
<td></td>
</tr>
<tr>
<td>D-LCL (IBS)</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>—</td>
</tr>
</tbody>
</table>

*Modified Rappaport histopathologic classification.
†Monoclonal antibodies obtained from Ortho (OKT), Becton-Dickinson, (Leu), or Coulter (B). Results reflect >70% positive immunofluorescent staining of fresh tumor cell suspensions.
‡Immunofluorescent staining of a rabbit anti-HeLa nucleolar protein antiserum on frozen sections from fresh lymph node biopsies. Positive reactivity indicates >60% of the cells showing nucleofluorescence within the morphological tumor cell population.

populations were then assayed for HMNA on cytocen-
tidifications, which appear to be very common in these
lesions.

**Studies on HMNA Expression in Normal Stimulated Human Lymphoid Cell Populations**

To further confirm our hypothesis that HMNA represents a marker of neoplastic lymphoid cells, we
studied a variety of human peripheral blood lymphoid cells that had been mitogen-stimulated, allogeneically
activated in vitro by mitogen and then maintained in
culture on T cell growth factor (TCGF) for several

Table 4. Karyotypic and HMNA Analysis of NHL Cells

<table>
<thead>
<tr>
<th>Lymphoma Type* (Cell Surface Ig†)</th>
<th>Karyotypic Abnormality‡</th>
<th>HMNA Reactivity§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large cell lymphoma B cell type (μχ +)</td>
<td>14q +, marker chromosome</td>
<td>+</td>
</tr>
<tr>
<td>Diffuse poorly differentiated lymphocytic, B cell type (μγχ +)</td>
<td>14q +</td>
<td>+</td>
</tr>
<tr>
<td>Nodular poorly differentiated lymphocytic, B cell type (μχ +)</td>
<td>14q +</td>
<td>+</td>
</tr>
<tr>
<td>Undifferentiated non-Burkitt's type (μδχ +)</td>
<td>13q+, 14q+, 11q−, abnormal chromosome 1</td>
<td>+</td>
</tr>
</tbody>
</table>

*Modified Rappaport classification, determined on H&E-stained paraffin-embedded sections from the same specimen.
†Cell surface Ig isotype was determined by immunofluorescence.
‡Cell suspensions at 10^6/ml were prepared in hypotonic saline
in the presence of Colcemid. The cells were then fixed in
Carnoy’s solution, and air-dried smears were prepared and G-banded. At
least 6 chromosome spreads were analyzed in each case.
§Tumor-cell-enriched suspensions were assayed for HMNA by immunofluorescence.
were then washed and assayed on cytocentrifuge preps as above. Different donors for 96 hr at 37°C in 5% CO2. The cells PBMC from 2 fixation. Which were assayed by indirect immunofluorescence after methanol at 37°C in 5% CO2. washed in PBS, and cytocentrifuged onto slides, on fixed cytocentrifuge preparations. Obtained by Ficoll-Hypaque separation.

Propagated on DEAE-purified IL-2 (TCGF) obtained from PHA-stimulated weeks. As seen in Table 5 and Fig. 2, none of the activated lymphoid cells of T or B lineage expressed the HMNA, even after culture in vitro for 21 days. DISCUSSION

As tumors of the immune system, NHL are among the most studied tumors. In recent years, numerous studies have appeared that attempted to correlate histopathologic morphological classification with cell surface phenotype.18,19 These studies were initially performed with conventional immunologic markers (e.g., En rosettes, Slg, etc.), and more recently, with monoclonal antibodies,20,21 they have established the apparent lymphoid cell lineage of most of the NHL. Unfortunately, with the exception of the use of specific antiidiotyp antibodies in Slg+ B cell NHL,22 none of the phenotyping reagents are able to discriminate between normal or neoplastic lymphoid cells that express a similar cell surface antigenic profile. This creates a number of problems for the pathologist, who must rely on morphological parameters alone to distinguish the malignant lymphoid cells, which frequently do not exhibit striking cellular atypia, from the normal or reactive lymphoid cell populations always present in lymphomatous lesions. Perhaps even more troublesome are the so-called borderline lesions, often referred to semidescriptively as “atypical hyperplasia” or by other equally ambiguous nomenclature. In these cases, there is architectural effacement of the lymphoid tissue with hyperplastic proliferative patterns of the lymphoid cells, but without marked cytologic atypia or the emergence of a definable monoclonal cell population.

In such cases, a marker corresponding to malignancy would be particularly useful. The HMNA, identified now in a variety of human tumors of epithelial, mesenchymal, as well as hematopoietic cell types,23,24 would appear to fulfill this criterion.

Also, the HMNA holds significant potential for experimental studies on the neoplastic lymphoid cell populations in NHL. As virtually all lymphoma cell suspensions from histopathologically involved lymph nodes, spleens, etc., contain variable numbers of putatively normal or reactive lymphoid cells, the HMNA provides a suitable marker for evaluating the relative purity of the tumor cell population after selective removal of contaminating reactive lymphoid cells.

An important question, of course, is: what are the anti-HMNA antisera recognizing, and what is its relationship to neoplastic transformation in human lymphoid cells? Studies done by Busch’s group in Namalwa Burkitt lymphoma cells, as well as in non-lymphoid cells, indicate that two antigenic moieties are identified.26 The HMNA antigens have molecular weights of approximately 68,000 and 61,000 daltons and have been shown to be electrophoretically homogeneous on two-dimensional polyacrylamide gels.27 Although not found in some normal cells, these antigens were also found in human embryonic fibroblasts, suggesting that they may represent oncofetal-like antigens. Moreover, it is possible that HMNA is present in normal tissue, but in quantities too minute to be detected in an immunofluorescence assay. Using an enzyme-linked immunoabsorbent assay, Kelsey et al.29 estimated that the concentration of HMNA present in extracts from tumor tissues was at least 30 times the background level of detection in extracts from normal tissues.

The putative false positive case of lymph node hyperplasia in an AIDS patient raises a number of interesting questions. This patient was thought to have lymphoma clinically and has continued to have marked diffuse lymphadenopathy for 8 mo. Whether his condition represents an incipient neoplastic lymphoid proliferation that has not as yet become morphologically discernible remains to be answered. We have now studied two other AIDS patients with diffuse lymphadenopathy showing the characteristic hyperplastic pattern seen in these patients, but who have been negative for HMNA.

Although there are still many questions to be answered about the biochemical nature and functional significance of the nucleolar antigen(s) associated with malignancy, at this time, HMNA appears to be an effective tumor cell marker for lymphoid cells and, as such, should be of considerable importance both diagnostically and biologically in the study of human lymphoma.
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Identification of human lymphoma cells by antisera to malignancy-associated nucleolar antigens

RJ Ford, M Cramer and FM Davis